GENETIC TESTING

The present invention relates to methods for the genetic testing of samples to determine the presence of Vascular Endothelial Growth Factor (VEGF) polymorphisms which are linked to a genetic predisposition to particular diseases or disorders.

VEGF, also known as vascular permeability factor (VPF) is a disulphide-linked homodimer of 34-42kDa. It is a potent endothelial cell mitogen and promotes angiogenesis (i.e. the formation of new blood capillaries from existing vessels). The critical importance of VEGF to angiogenesis is illustrated by studies of tumour models employing antibodies which neutralise VEGF, prevent neovascularisation and thereby inhibit tumour growth and metastasis.

VEGF also displays vascular permeability enhancing properties. VEGF is one of the most potent modulators of capillary permeability (50,000 times more active than histamine on a molar basis) as evidenced by intradermal injection of VEGF into guinea pig skin, which induces significant capillary permeability as measured in the Miles assay. In addition to skin, other vascular beds that respond to VEGF include the peritoneal wall, mesentery, diaphragm, cremaster muscle, lung and brain. Injection of VEGF rapidly induces permeability changes, which are often evident within minutes of injection. The effect is transient lasting 30-60 minutes and, importantly, it does not induce detectable injury to endothelium.

The gene for VEGF is found on chromosome 6 in humans and has 5' and 3' regulatory sequences which modulate transcription. Hypoxia, cytokine, endotoxin and hormonal control mechanisms are known to induce gene expression of VEGF.

VEGF-induced angiogenesis and VEGF-induced vascular permeability are major and critical components of a number of disease pathologies. Examples of such conditions are described below.

Proteinuria

VEGF mRNA and protein are colocalized in glomerular epithelia and collecting ducts in human fetal (6-24 weeks) and adult kidney. Furthermore KDR and Flt-1 receptor (receptors for VEGF) mRNA are similarly present in glomerular endothelium and peritubular capillaries in fetal and adult kidney. The VEGF-VEGF receptor system is important in renal ontogenesis and its constitutive expression in the adult kidney is required for normal physiological function.

Minimal Change Nephrotic Syndrome (MCNS) is the commonest cause of the nephrotic syndrome in children and up to 50% of affected individuals follow a frequently relapsing course. Heavy proteinuria is highly selective and often develops acutely over 24-48 hours. Clinical observation has linked a potential immune trigger for such relapses as upper respiratory tract infections often precede attacks and approximately 30% of cases have clinical manifestations of allergy. That an immune mechanism is central to the disease process is supported by the ability of immunosuppressive drugs, such as cyclosporin, high dose steroids, and cyclophosphamide, to produce disease remission. Renal biopsy, rarely performed coincident with onset of proteinuria, shows no evidence of a conventional classical immunological inflammatory mechanism involving antibody and complement deposition or interstitial cell infiltration. We have recently shown that VEGF is not the circulating vascular permeability factor described by others. However, increased glomerular podocyte expression of VEGF mRNA is associated with MCNS.

VEGF and cancer

There is substantial evidence that tumour growth, invasion and metastasis are angiogenesis-dependent. VEGF is commonly found expressed at high levels in

tumours including breast, bladder, colon and ovary. Increased immunostaining for VEGF has been documented adjacent to tumours in pre-existing vessels which were hyperpermeable, whereas vessels greater than 0.5mm distant from the tumour did not stain for VEGF and were not hyperpermeable.

The hypoxic conditions pertaining in tumours have been recognised as a major stimulant to induction of VEGF mRNA. Evidence suggests that in hypoxic regions of solid tumours, VEGF expression is regulated by hypoxia inducible factor-1 (HIF-1) a critical part of the oxygen regulated gene control system. In addition, of the most commonly encountered genetic changes detected in human cancer, the expression of mutant ras oncogenes, appear to enhance the expression of VEGF whereas there is no evidence between loss of p53 activity and VEGF expression. Low levels of VEGF measured in the primary tumours of lymph node negative breast cancer patients have been found to be prognostic for both relapse-free and overall survival. Others have associated intra-tumour VEGF concentration with level of angiogenesis as measured by microvessel density in breast cancer and higher levels in malignant compared to non-malignant tissue but could not confirm the prognostic significance in their patient population. In general, it appears there is an association between microvessel density and VEGF expression in breast cancer, and also in colon, gastric, vulvar and oesophageal squamous cell carcinoma. An experimental model of human melanoma SK-MEL-2 cells grown in immunodeficient mice clearly shows that VEGF promotes melanoma growth by stimulating angiogenesis and metastasis. Wild type cells formed small poorly vascularized tumours consistent with minimal expression of constitutive and hypoxia-inducible VEGF. Stably transfected cells with sense cDNA for VEGF expressed and secreted large amounts of VEGF and formed well-vascularized tumours with minimal necrosis, whereas antisense (VEGF) cDNA transfected cells produced no detectable VEGF and formed small minimally vascularized tumours with extensive necrosis.

VEGF and inflammatory joint disease

VEGF promotes the pathophysiological process of angiogenesis in the joint, which could be important both to susceptibility, and severity of disease. Significantly high levels of VEGF are present in inflammatory synovial fluids from Rheumatoid Arthritis (RA) patients. Interestingly, the VEGF in synovial fluid (SF) is not complexed to soluble receptor and as it binds in a receptor capture assay, it is likely to be bioactive. Additionally, the second described member of the VEGF family, Placenta Growth Factor (PlGF) is also present in SF at high levels in some patients either as the homodimer or as a heterdimer with VEGF. This may have functional implications as PlGF is reported to synergise and enhance the bioactivity of VEGF. Plasma levels of VEGF in patients with RA are not increased indicating that cells from within the joint secrete the VEGF in SF. Neutrophils are a source of VEGF which is secreted on cell activation and there is a significant correlation between neutrophil cell counts and VEGF concentrations in SF. Macrophages in the synovium stain positive for VEGF and others report synovial fibroblasts as a potential source of VEGF. Thus it is likely that several cell types contribute to the high SF level of VEGF.

VEGF and diabetes

Increased levels of VEGF and PlGF have been demonstrated in diabetic retinopathy, the commonest cause of blindness in the working population. Experimental models support the role of VEGF in promoting retinal neovascularisation which can be inhibited using soluble VEGF-receptor chimeric proteins. Hypoxia-induced expression of VEGF is a likely mechanism in a number of neovascularizing ocular diseases. However, in diabetic retinopathy, it is possible that high glucose levels and advanced glycation end products contribute to the upregulation of VEGF expression and progression of the retinal angiogenesis

There is a need for reliable tests for diagnosing diseases and disorders which are associated with abnormal VEGF levels. One way by which this may be done

involves genetic testing. Genetic testing may be defined as the analytical testing of a subject's nucleic acid to determine if the DNA of a patient contains mutations (or polymorphisms) that either cause or increase susceptibility to a disease state or disorder or are in "linkage" with the gene causing a disease state and are thus potentially indicative of a predisposition to the disease state or disorder.

The early detection of a predisposition to a disease or disorder presents the best opportunity for medical intervention. Early genetic identification of risk may improve the prognosis for a patient through early intervention before clinical symptoms of the disease or disorder manifest.

In cases where patients with similar symptoms are treated with variable success with the same therapeutics, genetic testing may differentiate patients with a genetic rather than developmental basis for their symptoms, thus leading to the potential need for different approaches to therapy.

It is an aim of the present invention to provide methods for genetic screening of polymorphisms associated with the VEGF gene for the diagnosis of, or as an indication of a risk or predisposition to, diseases or disorders associated with abnormal VEGF expression.

According to a first aspect of the present invention there is provided an *in vitro* method for diagnosing or detecting a predisposition to a disease or disorder associated with abnormal Vascular Endothelial Growth Factor gene expression, the method comprising examining regulatory elements associated with the Vascular Endothelial Growth Factor gene to detect the presence of a genetic polymorphism which is linked to the disease or disorder.

By "polymorphism" we mean a different gene sequence from the wild type. Polymorphisms can be variants that are generally found between individuals of

different ethnic backgrounds or from different geographical areas and which do not affect the function of the gene. Other polymorphisms are those which lead to differences in the function of the gene or may produce an inactive gene product or may modulate the production of the gene product. "Polymorphisms linked to the disease or disorder" are polymorphisms which occur with more frequency in subjects which develop a disease or disorder.

By "gene" we mean all coding sequences between the start and stop codon of the VEGF gene (including introns and exons).

By "regulatory elements" we mean the DNA that is 5' and 3' of the gene and which is involved in regulating gene transcription. For instance, transcription factor binding sequences, the TATA box, the 5' promoter and 5' and 3' untranslated regions (UTRs). This definition also encompasses the DNA 5' of the first codon of the first Exon of the VEGF gene.

The method according to the first aspect of the invention allows an investigator to identify subjects expressing a genetic polymorphism indicative of diseases or disorders associated with abnormal VEGF gene expression or a predisposition to such diseases or disorders. Identification of such a polymorphism allows the investigator to determine those patients who have or are more at risk of developing a VEGF associated disease or disorder. This enables a medical practitioner to take appropriate action to prevent or lessen the likelihood of onset of the disease or disorder or to allow appropriate treatment of the disease or disorder.

The invention is based on the discovery of particular genetic polymorphism patterns for the VEGF gene that were found to be associated with certain diseases or disorders in which the wild type VEGF gene plays a role. The inventors established this fact by correlating information relating to VEGF polymorphisms (identified by their experiments) with the medical status of subjects from which the test sample was

derived. Furthermore, given the identification of such polymorphisms it is possible, according to the method of the first aspect of the invention, to correlate the frequency of a polymorphism in the population with the prevalence of any disease or disorder. Thus diseases or disorders which had not previously been associated with VEGF may be identified as therapeutic targets for VEGF modulators.

Whilst not wishing to be bound by any hypothesis the inventors believe that particular VEGF polymorphisms are indicative of a predisposition to abnormal VEGF gene expression that has an effect upon the etiology of the disease state. Furthermore we believe that VEGF expression in man has genetic control through the existence of polymorphisms in the VEGF gene and its regulatory elements. This control will be manifest through the ability to produce or express high or low amounts of biologically active VEGF to a given stimulus which might include infection (endotoxin), hypoxia (low oxygen tension), cytokines (TNFα, TGFβ1, PlGF), hormones (oestrogen) etc. Similarly, response to agents intended to suppress VEGF may also vary according to genetic polymorphism status, using drugs such as dexamethasone and other steroids. We believe that genetic polymorphisms in the promoter, 5' and 3' untranslated regions and the translated regions of VEGF gene may control an individual's response to one or more of these stimuli and, therefore, act as a diagnostic marker of disease susceptibility, response to treatment or disease prognosis.

We have found that VEGF polymorphisms may be linked with diseases in which there is high VEGF expression. Examples of such conditions include:

1. Cancer - in particular highly vascularised tumours, metastatic cancer in comparison to primary non metastatic cancer

2. Inflammatory joint disease, in particular rheumatoid arthritis but also other arthritides

3. Proteinuric disease

4. Diabetes - particularly the complications of retinopathy and nephropathy

5. Hyper Ovarian Stimulation Syndrome

6. Bleeding disorders linked to female reproductive cycle

We have also established that the existence of VEGF polymorphisms may be linked with diseases associated with low VEGF expression. These conditions include:

1.Ischaemic heart disease (high risk of fatality)

2. Infertility linked to failure of implantation

3. Poor wound healing and vascular ulceration

4. Systemic sclerosis

It will be appreciated that correlations will exist with other diseases or disorders associated with abnormal VEGF expression (e.g. diabetes and particularly diabetic complications such as retinopathy). These correlations may be used to calculate the chances of an individual with a particular polymorphism contracting the disease or disorder, used to aid diagnosis or predict prognosis according to the method of the first aspect of the present invention.

The inventors have performed experiments to screen the VEGF gene and its regulatory elements for polymorphisms which may be linked to VEGF related diseases. Having established such links the inventors have established that DNA taken from a subject may be analysed to help establish a diagnosis of the above mentioned conditions or to establish whether or not a subject is predisposed to develop such a condition.

Various polymorphisms have been identified by the inventors which may be linked to the diseases or disorders and detected according to the first aspect of the invention (see Example 1). It will be appreciated that specific polymorphims may be linked to specific conditions.

A preferred polymorphism which may be detected according to the first aspect of the invention is located 460 bases 5' of the transcription start for VEGF (- 460) and

represents a point mutation of a Cytosine nucleotide (C) to a Thymine nucleotide (T)). Genotypes CC, CT and TT were identified for this polymorphism.

We have found that the - 460 polymorphism (i.e. C to T) is significantly over-represented in patients with Ischaemic Heart Disease compared with angiographically normal individuals whereas the CC homozygous genotype indicates that an individual is protected from developing Ischaemic Heart Disease. This indicates that VEGF has a central role to play in the ischaemic disease process. Furthermore, subjects who have Thymine at - 460 may have an increased susceptability to developing heart disease and/or may need extra attention if suffering from a heart condition. Therefore the presence of the polymorphism may be used as the basis of giving life style recommendations (to those who may be prone to developing the disease) and will also be relevant in the diagnosis and therapeutic approach to those with heart disease.

We have also found that a CC homozygous genotype at –460 indicates that an individual is predisposed to develop rheumatoid arthritis or ovarian cancer (see the Examples). Furthermore, a TT homozygous genotype at –460 indicates that an individual is predisposed to develop Nephrotic Syndrome.

Another preferred polymorphism which may be detected according to the first aspect of the invention is located 405 bases 3' of the transcription start for VEGF (+ 405). This polymorphism is a point mutation of a Guanine nucleotide (G) to a Cytosine nucleotide (C)). Genotypes GG, GC or CC were identified for this polymorphism.

We have found that the CC homozygous genotype is significantly under-represented in patients with rheumatoid arthritis compared with normal individuals. This indicates that subjects who have the GG genotype at +405 indicates that an individual is predisposed to develop rheumatoid arthritis. Therefore the presence of the polymorphism may be used as the basis of giving life style recommendations (to those

who may be prone to developing the disease) and will also be relevant in the diagnosis and therapeutic approach to those with rheumatoid arthritis.

The – 460 and + 405 polymorphisms are particularly indicative of an increased susceptibility to the diseases or disorders listed above. In particular we have established an association of these two polymorphisms with steroid sensitive nephrotic syndrome in children and also ischaemic heart disease, rheumatoid arthritis, and ovarian cancer. The identification of such polymorphisms helps in the diagnosis of such conditions.

The inventors established that VEGF polymorphisms may be identified and used as an indicator of a predisposition to a disease state by performing molecular biological studies on the VEGF gene. The techniques and experiments performed are detail below and in Example 1.

1. IDENTIFICATION OF VEGF POLYMORPHISMS

Techniques for determining the presence of a VEGF genetic polymorphism may be nucleic acid techniques based on size or sequence (e.g. nucleic acid sequencing or restriction fragment length polymorphism (RFLP)) or hybridisation techniques. These techniques may also involve amplifying the nucleic acid before analysis. Suitable amplification techniques include cloning and polymerase chain reaction (PCR). Amplification products may be assayed in a variety of ways, including detecting specific tagged oligonucleotide primers in the reaction products, size analysis, restriction digestion followed by size analysis, allele-specific oligonucleotide hybridisation and sequencing.

The inventors discovered VEGF polymorphisms using the polymerase chain reaction (PCR) to amplify at least a fragment of the VEGF gene and its regulatory elements. A sample of DNA from a normal subject was subjected to PCR amplification using PCR primers specific for the region around a putative polymorphic

fragment of DNA only. A region of around 200 - 300 bases was amplified from the VEGF gene or its regulatory elements using suitable PCR primers.

PCR-SSCP (single-stranded conformation polymorphism) is a preferred technique for the initial discovery of DNA sequence polymorphisms. A typical strategy for performing PCR-SSCP is outlined below and also in Example 1.

Single-Stranded Conformation Polymorphism (SSCP) Analysis

A) Primer Design and PCR Amplification

(i) Specific primers were designed to amplify DNA segments of 200 - 300 base pairs in length.

By way of example only, PCR primers suitable for amplifying a region around the +405 polymorphism are listed below as SEQ ID No. 1 and SEQ ID No. 2.

Forward primer: 5'ATTTATTTTTGCTTGCCATT 3'(SEQ ID No. 1)

Reverse primer: 5'GTCTGTCTGTCTGTCCGTCA3' (SEQ ID No. 2)

PCR primers suitable for amplifying a region around the –460 polymorphism are listed below as SEQ ID No. 3 and SEQ ID No. 4.

Forward primer: 5' CTC GGC CAC CAC AGG GAA GC 3' (SEQ ID No. 3)

Reverse primer: 5' TAC GTG CGG ACA GGG CCT GA 3' (SEQ ID No. 4)

(ii) Amplification was carried out in a 20µl reaction containing 100ng of genomic DNA; 10 pmoles of each primer; 0.75mM of each dNTP; 10% DMSO; 4mM $MgCl_2$ (for both primer pairs); 16.6mM $(NH_4)_2SO_4$; 67mM Tris.HCl pH 8.0; 85mg/ml BSA and 0.5U of Taq DNA polymerase. Samples were denatured at 95°C for 3 min.

followed by 32 cycles at 95°C for 1 min., 55 or 65°C* for 1 min. (* for +405 and –460 respectively), 72°C for 1min., with a final extension step of 72°C for 5 min.

B) SSCP Analysis

(i) 5µl of each PCR product and 2µl of formamide dye (98% formamide, 10mM EDTA, 0.025% bromophenol blue) were denatured at 95°C for 5 min. then placed in ice.

(ii) Samples were then electrophoresed through a 10% 1 x TBE 39:1 acrylamide : bis-acrylamide 20 x 20cm gel in 1 x TBE running buffer at 10°C for 16 hours at 10-14mA depending on PCR product length. Banding patterns were visualised by silver staining of the gels. Briefly gels were fixed for 15 min. in 10% ethanol, 0.5% acetic acid; stained for 30 min. in 0.15% silver nitrate solution; then allow to develop for 15 min in 1.5% sodium hydroxide, 0.25% formaldehyde with distilled water rinses between each step. The stained gels were then fixed in 0.75% sodium carbonate solution rinsed in water and dried down.

A direct sequencing strategy may also be utilised for discovering polymorphisms. An example of such a strategy is outlined below.

Direct Sequencing

A) PCR Amplification

Primers and PCR amplification was as described for PCR- SSCP above.

B) PCR Product Purification

PCR products were purified to remove excess dNTPS and primers using Amersham Pharmacia Biotech GFX™ columns as per the manufacturer's instructions.

C) Direct fluorescent PCR-cycle sequencing

The purified PCR products were then directly cycle-sequenced in both the forward and reverse orientation, employing the original PCR primers, using the Cy5™ Thermo Sequenase™ dye terminator kit (Amersham Pharmacia Biotech) as per the manufacturer's instructions. Sequencing reactions were analysed using the ALFexpress™ automated DNA sequencer (Amersham Pharmacia Biotech).

2. CALCULATION OF ALLELE FREQUENCIES

Following identification and sequencing of polymorphisms, the allele frequencies of the polymorphisms identified within the normal population were calculated.

Given the discovery of VEGF polymorphisms which may be linked to changes in VEGF expression, the inventors proceeded to detect the polymorphisms in populations of subjects with various diseases or disorders in order that genetic linkage with the disease or disorder may be established (see Examples 2 and 3). The existence of such linkage allows a sample from an individual to be used for diagnosis, or detection of a predisposition to, a disease or disorder associated with abnormal Vascular Endothelial Growth Factor gene expression according to the first aspect of the invention (see Example 4).

3. DETECTION OF POLYMORPHISMS ACCORDING TO THE METHOD OF THIS INVENTION

Various strategies may be used to detect polymorphisms according to the method of the invention.

A technique involving Restriction Enzyme Digestion (RED) is a preferred technique for detection of polymorphisms according to the method of the invention and is outlined in more detail in Example 2.

This RED technique is based upon the fact that polymorphisms can lead to the production of different sized DNA fragments following treatment with a restriction enzyme (because of the introduction or deletion of a restriction site by the mutation causing the polymorphism). These fragments may be visualised on gels and the polymorphism identified based upon the number and size (i.e. distance moved on the gel) of the fragments from a DNA sample derived from a subject.

Preferably the DNA used in the RED technique is amplified prior to the restriction enzyme digestion (e.g. as outlined above in the methods for discovering polymorphisms).

In the situation that neither the wild type nor the mutant allele abolishes or introduces a restriction enzyme site, a restriction enzyme site may be introduced by specifically designing PCR primers which introduce restriction sites into the amplified product. The introduced enzyme site allows differentiation between polymorphic alleles and wild type by size analysis. For example if the restriction products of the amplified product are analysed by gel electrophoresis (agarose or polyacrylamide gel, for example) the alleles with the introduced restriction enzyme site will produce an extra band on the gel.

Details of other preferred techniques that may be employed according to the method of the invention are outlined more fully below.

(i) HETERODUPLEX AND SINGLE-STRANDED CONFORMATION POLYMORPHISM (SSCP) ANALYSIS

A) Primer Design and PCR Amplification

Primers were designed to amplify DNA segments of 200 - 300 base pairs in length. The primers used for the G → C base change at +405 were the primers identified as SEQ ID No 1 and SEQ ID No 2. The primers used for the C → T base change at –460 were the primers identified as SEQ ID No 3 and SEQ ID No 4.

Amplification was carried out in a 20µl reaction containing 100ng of genomic DNA; 10 pmoles of each primer; 0.75mM of each dNTP; 10% DMSO; 4mM $MgCl_2$ (for both primer pairs); 16.6mM $(NH_4)_2SO_4$; 67mM Tris.HCl pH 8.0; 85mg/ml BSA and 0.5U of *Taq* DNA polymerase. Samples were denatured at 95°C for 3 min. followed by 32 cycles at 95°C for 1 min., 55 or 65°C* for 1 min. (* for +405 and –460 respectively), 72°C for 1min., with a final extension step of 72°C for 5 min.

B) Heteroduplex and SSCP Analysis

5µl of each PCR product and 2µl of formamide dye (98% formamide, 10mM EDTA, 0.025% bromophenol blue) were denatured at 95°C for 5 min. then placed in ice. Samples were then electrophoresed through a 10% 1 x TBE 39:1 acrylamide : bis-acrylamide 20 x 20cm gel in 1 x TBE running buffer at 10°C for 16 hours at 10-14mA depending on PCR product length. Banding patterns were visualised by silver staining of the gels. Briefly gels were fixed for 15 min. in 10% ethanol, 0.5% acetic acid; stained for 30 min. in 0.15% silver nitrate solution; then allow to develop for 15 min in 1.5% sodium hydroxide, 0.25% formaldehyde with distilled water rinses between each step. The stained gels were then fixed in 0.75% sodium carbonate solution rinsed in water and dried down.

(ii) DIRECT SEQUENCING

A) PCR Amplification

Primers and PCR amplification was as described in the section on heteroduplex and single-stranded conformation polymorphism analysis (SSCP).

B) PCR Product Purification

PCR products were purified to remove excess dNTPS and primers using Amersham Pharmacia Biotech GFX™ columns as per the manufacturer's instructions. PCR products may also be purified using other types of size exclusion columns, enzymatically using shrimp alkaline phosphatase and exonuclease I and using

magnetic streptavidin-coated beads (Dynal Ltd.) and biotin labelled PCR primers in the initial amplification.

C) Direct fluorescent PCR-cycle sequencing

The purified PCR products were then directly cycle-sequenced in both the forward and reverse orientation, employing the original PCR primers, using the Cy5™ Thermo Sequenase™ dye terminator kit (Amersham Pharmacia Biotech) as per the manufacturer's instructions. Sequencing reactions were analysed using the ALFexpress™ automated DNA sequencer (Amersham Pharmacia Biotech).

PCR products may also be sequenced directly by dye-labelled primer cycle-sequencing using the Amersham Thermo Sequenase™ fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) or the Autoload sequencing kit (Amersham Pharmacia Biotech) as per the manufacturer's instructions.

(iii) SEQUENCE SPECIFIC OLIGONUCLEOTIDE HYBRIDIZATION (SSO)

A) PCR Amplification

Primers and PCR amplification were as described in the section on heteroduplex and single-stranded conformation polymorphism analysis (SSCP).

B) Dot or Slot Blotting of PCR Products

If there is paraffin oil covering the amplified products it was extracted from the aqueous phase of the reaction using a mixture of 200µl TE and 200µl chloroform. A 15µl aliquot of the extracted products (1/15th of the original reaction) was blotted onto Hybond N+ nylon membranes (Amersham Ltd) using a 48 well slot-blot vacuum manifold (Hoefer Scientific Instruments) or a 96 well dot-blot manifold (Life Technologies Ltd.). Wells were washed with 200µl TE before the filters were air-dried on filter paper. Fixation of the DNA was achieved by transferring the filters to sheets of filter paper soaked in 0.4M NaOH for 15 mins. The filters were then transferred to neutralising solution for 15 mins, washed in 2x SSC for 5 mins before being air-dried as previously. Two positions on each filter were reserved for the blotting of amplified DNA to serve as positive and negative hybridisation controls for each oligonucleotide probe used.

C) Hybridisation with Labelled Probes

For each polymorphism, oligonucleotide probes were designed which encompassed the polymorphic site, with each probe being specific for each variant base.

A 10pmol aliquot of each oligonucleotide probe stock was radioactively labelled with ^{32}P-ATP (10µCi/µl) at the 5' terminus using T4 polynucleotide kinase (Promega Ltd.). Alternatively, probes were non-radioactively labelled at their 3' terminus with Digoxygenin-ddUTP (DIG) using terminal deoxynucleotidyl

transferase according to manufacturers' instructions (BCL). Dot/slot blotted filters were pre-hybridized for at least 1 hour at 58°C in hybridization buffer before their transfer to 5ml hybridization buffer containing the labelled oligonucleotide probe. Hybridizations were carried out for a minimum of 4 hours in sealed chambers using 1pmol of probe per ml hybridisation buffer with incubation at the probe T_m calculated for each probe.

Following hybridization, filters were washed in 2x SSC, 0.1% SDS for 15 minutes with gentle agitation at room temperature. Filters were then transferred to tetramethyl ammonium chloride (TMAC) wash solution pre-warmed to the appropriate washing temperature and washed with gentle agitation for 15 minutes at the calculated washing temperature. Filters were soaked briefly in 2x SSC to remove traces of TMAC which would otherwise have increased the background signal, and sealed between polythene sheets before direct autoradiography (^{32}P-labelled probes) or chemiluminescent detection and autoradiography (digoxygenin-ddUTP labelled probes).

Samples were judged to have a particular polymorphic base if they gave positive signals (compared to controls) with that specific oligonucleotide.

(iv) OTHER TECHNIQUES

Other techniques that may be used to detect polymorphisms according to the first aspect of the invention include :-

(1) Sequence Specific Priming (SSP) [also described as Amplification Refractory Mutation System (ARMS)];

(2) Mutation Scanning [e.g. using the PASSPORT™ Mutation Scanning Kit (Amersham Pharmacia Biotech)];

(3) Chemical Cleavage of Mismatch Analysis;

(4) Non-isotopic RNase Cleavage Assay (Ambion Ltd.);

(5) Enzyme Mismatch Cleavage Assay; and

(6) Single Nucleotide Extension Assay.

The method according to the first aspect of the invention is particularly suitable for being carried out on genomic DNA, particularly on isolated genomic DNA. Such genomic DNA may be isolated from blood or tissue samples or from other suitable sources using conventional methods. Preferably the DNA is isolated from whole blood or granulocytes (e.g. as described in Example 4).

An individual having a polymorphism in the VEGF gene or its regulatory elements may be identified using the abovementioned techniques. The identification of the polymorphism may be used to diagnose or predict a predisposition to developing the diseases or disorders according to the method of the first aspect of the present invention.

A prediction or diagnosis based upon the method according to the present invention depends upon a linkage being made between a particular disease or disorder and the specific polymorphism in question. Such linkage was established by the inventors by performing further experiments and making statistical analyses as described herein (see the Examples). Links between the +405 and –460 polymorphisms are discussed above and in the Examples. Provision of data based upon linkage analyis enables a clinician to interpret the significance of genotypes identified by sequencing DNA according to the method of the invention. The clinician may then make a judgement regarding the likelihood of a patient developing, or having, a particular disease or disorder. Such knowledge is important in the clinical management of specific diseases or disorders associated with abnormal VEGF gene expression. It will be appreciated that linkage data may be provided to a user of the method according to the invention (e.g. a technician or clinician) by incorporating a data sheet as part of a kit (see below).

The various elements required for a technician to perform the method of the first aspect of the invention may be incorporated in to a kit. Thus according to a second aspect of the present invention there is provided a kit comprising:

A) PCR primers for amplifying genetic polymorphisms in regulatory elements associated with the Vascular Endothelial Growth Factor gene that are linked to a disease or disorder; and

B) Control DNA samples of known genotype for each polymorphism.

It is preferred that the kit comprises primers specific for a target sequence of sample DNA known to contain a polymorphism of interest. Suitable PCR primers for the kit include SEQ ID No. 1 and SEQ ID No. 2 (for +405 polymorphism genotyping) or SEQ ID No. 3 and SEQ ID No. 5 (for -460 polymorphism genotyping).

The kit may further comprise:

C) a suitable restriction enzyme for generating fragments of the DNA sample;

D) a data card outlining linkage between a particular polymorphism and a disease;

E) protocols for PCR amplification, restriction enzyme digestion of PCR products and agarose gel electrophoresis of DNA fragments;

F) relevant buffers (e.g. A- D from Example 4).

Buffers provided with the Kit may be in liquid form and preferably provided as pre-measured aliquots. Alternatively the buffers may be in concentrated (or even powder form) for diluting.

The Kit may further comprise suitable reaction vessels, centrifuge tubes etc.

The invention will be further described, by way of example only, with reference to the accompanying drawings, in which:

Fig. 1 illustrates the positions of the – 460 and +405 polymorphisms in a gene sequence (SEQ ID NO. 6) representing the promoter, 5' Untranslated Region (UTR) and exon 1 of the VEGF gene;

Fig. 2 illustrates the association between the –460 (TT=9, TC=7, CC=5) and +405 (CC=3, CG=10, GG=8) genotypes and VEGF production (mean ± S.E.M.) from cultured PMBCs according to Example 1, (A) shows LPS stimulated VEGF production for I) the – 460 genotype and II) +405 genotype whereas as (B) shows colbalt chloride-stimulated VEGF production for I) the – 460 genotype and II) +405 genotype.

EXAMPLE 1 : IDENTIFICATION OF POLYMORPHISMS

Experiments were performed to locate polymorphisms within the 5' promoter region of the VEGF gene. We established that the VEGF gene is polymorphic and identified and sequenced at least 14 polymorphisms (see Table 1) in a group of 115 normal individuals.

The promoter and the whole of exon 1, containing the 5' untranslated region (UTR) and first coding region, of the VEGF gene (see Fig. 1) were screened for sequence polymorphisms using the polymerase chain reaction (PCR) to amplify regions of interest and single-stranded conformation polymorphism (SSCP) analysis to identify sequence variations. All PCR products exhibiting SSCP band shifts were directly analysed by automated fluorescent cycle sequencing to determine the sequence variations.

1.1 METHODS

1.1.1 Single-Stranded Conformation Polymorphism (SSCP) Analysis

A) Primer Design and PCR Amplification

(i) Specific primers were designed to amplify DNA segments of 200 - 300 base pairs in length in the VEGF gene or it's regulatory elements. Polymorphisms were identified from a range of amplified DNA sequences using a number of primer sets.

Examples of primers used:

1) Forward primer: 5'ATTTATTTTTGCTTGCCATT 3'(SEQ ID No. 1)

Reverse primer: 5'GTCTGTCTGTCTGTCCGTCA3' (SEQ ID No. 2)

These PCR primers amplified a region around the polymorphism subsequently located at + 405.

2) Forward primer: 5' CTC GGC CAC CAC AGG GAA GC 3'
(SEQ ID No. 3)

Reverse primer: 5' TAC GTG CGG ACA GGG CCT GA 3'
(SEQ ID No. 4)

These PCR primers amplified a region around the polymorphism subsequently located at – 460.

(ii) Amplification was carried out in a 20µl reaction containing 100ng of genomic DNA; 10 pmoles of each primer; 0.75mM of each dNTP; 10% DMSO; 4mM $MgCl_2$ (for both primer pairs); 16.6mM $(NH_4)_2SO_4$; 67mM Tris.HCl pH 8.0; 85mg/ml BSA and 0.5U of Taq DNA polymerase. Samples were denatured at 95°C for 3 min. followed by 32 cycles at 95°C for 1 min., 55 or 65°C* for 1 min. (* for +405 and –460 respectively), 72°C for 1min., with a final extension step of 72°C for 5 min.

B) SSCP Analysis

(i) 5µl of each PCR product and 2µl of formamide dye (98% formamide, 10mM EDTA, 0.025% bromophenol blue) were denatured at 95°C for 5 min. then placed in ice.

(ii) Samples were then electrophoresed through a 10% 1 x TBE 39:1 acrylamide : bis-acrylamide 20 x 20cm gel in 1 x TBE running buffer at 10°C for 16 hours at 10-14mA depending on PCR product length. Banding patterns were visualised by silver staining of the gels. Briefly gels were fixed for 15 min. in 10% ethanol, 0.5% acetic acid; stained for 30 min. in 0.15% silver nitrate solution; then allow to develop for 15 min in 1.5% sodium hydroxide, 0.25% formaldehyde with distilled water rinses between each step. The stained gels were then fixed in 0.75% sodium carbonate solution rinsed in water and dried down.

1.1.2 Direct Sequencing

A direct sequencing strategy was also utilised for discovering polymorphisms. An example of such a strategy is outlined below.

A) PCR Amplification

Primers and PCR amplification was as described for PCR- SSCP above.

B) PCR Product Purification

PCR products were purified to remove excess dNTPS and primers using Amersham Pharmacia Biotech GFX™ columns as per the manufacturer's instructions.

C) Direct fluorescent PCR-cycle sequencing

The purified PCR products were then directly cycle-sequenced in both the forward and reverse orientation, employing the original PCR primers, using the Cy5™ Thermo Sequenase™ dye terminator kit (Amersham Pharmacia Biotech) as per the manufacturer's instructions. Sequencing reactions were analysed using the ALFexpress™ automated DNA sequencer (Amersham Pharmacia Biotech).

1.1.3 Linkage analysis with VEGF production from normal cells

Following identification and sequencing of polymorphisms, the allele frequencies of the polymorphisms identified within the normal population were calculated.

Preliminary linkage experiments were performed to evaluate whether or not the polymorphisms could be linked to variations in VEGF produced by subjects. Peripheral Blood Mononuclear cell (PBMCs) were isolated by conventional means from a number of subjects and the amount of VEGF assayed under control conditions (unstimulated) and when activated by treatment with LPS (1 ng/ml) after 72 hours incubation or when activated with Cobalt Chloride. Levels of VEGF and the polymorphism exhibited by the subject were then correlated.

1.2 RESULTS

1.2.1 Polymorphisms

Table 1 summaries 14 polymorphisms located in DNA 5' of the first VEGF intron.

Table 1 :- VEGF gene sequence polymorphisms identified, with associated allele frequencies.

Sequence polymorphism	Position [b]	Allele frequencies in healthy individuals (N=115)	
G → C	+405	0.709	0.291
T insertion [a]	+183/+191	0.996	0.004
T insertion [a]	-26/-35	0.996	0.004
C → A	-37	0.983	0.017
A → C	-141	0.935	0.065
G → A [d]	-152	0.983	0.017
C → T [d]	-160	0.987	0.013
C → T [a]	-165	0.996	0.004
C → A	-172	0.987	0.013
AG deletion	-247/-250	0.991	0.009
C → T	-460	0.526	0.474
C → A	-914	0.996	0.004
G insertion [c]	-1045/-1051	0.987	0.013
C → A [c]	-1117	0.987	0.013

a – only observed in 1 individual **b** – transcription start = +1
c and **d** – polymorphisms together

Some of these polymorphisms are rare; a C to A base change at nucleotide –37, a C to A base change at nucleotide –172, and AG deletion between –247 and –250 and a T insertion between –26 and –35. The less common alleles for these polymorphisms have allele frequencies in the normal population of less than 0.05

1.2.2 Linkage analysis with VEGF production from normal cells

For the +405 polymorphism there was a significant correlation between genotype and LPS-stimulated PBMC VEGF protein production (Spearman correlation [two-tailed] r = 0.4951, p = 0.0225). Highest VEGF production (mean ± S.E.M.) was observed for the GG genotype (N = 8) (660.7 pg/ml ± 114.5), intermediate production was observed for the GC genotype (N = 10) (537.4 pg/ml ± 73.4) and lowest production for the CC genotype (N = 3) (262.6 pg/ml ± 99.5) (see Fig. 2).

EXAMPLE 2

Experiments were performed on samples taken from individuals with various medical conditions to establish linkage between the polymorphisms identified in Example 1 and relevant disease conditions. The Restriction Enzyme Digestion technique was used to detect the relevant polymorphisms.

Of the polymorphisms identified in Example 1, the – 460 and + 405 were identified as good candidates for linking to specific diseases or disorders and further experiments were conducted with these specific polymorphisms.

2.1 METHODS

2.1.1 Primer Design and Restriction Enzyme Information

When experiments were performed to identify polymorphisms in individuals, the PCR primers were designed to amplify the regions putatively encompassing the base variations.

Where a base change was observed to create or delete a restriction endonuclease site, primers were re-designed to allow for polymorphism typing of individuals using restriction enzyme digestion and agarose gel electrophoresis. If no restriction site was present at the site of the base variation one was created by a single base alteration within the forward amplification primer.

By way of illustration only, this Example relates to PCR (and therefore specific primers) designed for detecting polymorphisms at – 460 and + 405 (see above).

For the C → T base change at –460 a single base alteration was incorporated within the forward amplification primer to create a BstUI site when the C allele was present. This introduction allowed differentiation between the DNA fragments generated during the restriction enzyme treatment of DNA samples from the different allelles.

Table 2: Primer design for detecting the –460 and +405 polymorphisms

Sequence polymorphism	Primers	Restriction enzyme used to detect polymorphism	Allele	DNA fragment sizes
G → C At +405	F 5'ATTTATTTTTGCTTGCCATT 3' (SEQ ID No. 1) R 5'GTCTGTCTGTCTGTCCGTCA3' (SEQ ID No. 2)	BsmFI	G C	193bp, 111bp 304bp
C → T At –460	*F 5'TGTGCGTGTGGGGTTGAGCG 3' (SEQ ID No. 5) R 5' TACGTGCGGACAGGGCCTGA 3' (SEQ ID No. 4)	BstUI	C T	155bp, 20bp 175bp

The Forward –460 primer (SEQ ID No. 5) had a C – base change to create BstUI restriction site

2.1.2 PCR Amplification

Amplification was carried out in a 20µl reaction containing 100ng of genomic DNA; 10 pmoles of each primer; 0.75mM of each dNTP; 10% DMSO; 4 - 5mM* $MgCl_2$ (* for +405 and –460 respectively); 16.6mM $(NH_4)_2SO_4$; 67mM Tris.HCl pH 8.0; 85mg/ml BSA and 0.5U of *Taq* DNA polymerase. Samples were denatured at 95°C for 3 min. followed by 32 cycles at 95°C for 1 min., 55 or 60°C* for 1 min. (* for +405 and –460 respectively), 72°C for 1min., with a final extension step of 72°C for 5 min.

2.1.3 Restriction Enzyme Digestion

5µl of each PCR product was checked on an agarose gel and if amplification was satisfactory the 15µl of sample was digested by addition of 2 µl of 10 x restriction buffer, 0.2 µl of BSA (at 10mg/ µl), 2 - 5 units (i.e. 0.2 - 0.5µl at 10U/µl) of the appropriate restriction enzyme and dH_2O to 20µl. Samples were incubated at the prescribed temperature for the particular restriction enzyme for 2-3 hours.

2.1.4 Agarose Gel Electrophoresis

To determine the restriction digest pattern for each sample, samples containing 4µl of 5 x TBE loading dye were electrophoresed at 100-150v for 1-2 hours in 1 x TBE buffer (0.89M Tris, 0.89M orthoboric acid, 20mM EDTA) through a 1 X TBE 3% agarose gel containing ethidium bromide (at 10mg/ml) with the inclusion of a 100bp ladder (GIBCO-BRL). The gel was visualised on a UV trans-illuminator and photographed.

2.1.5 Linkage Analysis

The prevalence of the polymorphisms was correlated with medical conditions identified in populations of subjects from which the DNA was derived. Suitable statistical analysis was performed.

2.2 RESULTS

2.2.1. ISCHAEMIC HEART DISEASE

Significant differences were identified in the prevalence of the – 460 polymorphism in ischaemic and non-ischaemic populations.

The -460 polymorphism

Blood was drawn from subjects at the time of an angiographic study, and DNA extracted by standard techniques. Angiographic normality was defined *a priori* as absence of atheromatous coronary stenosis > 30% of luminal diameter in any epicardial coronary artery.

The area of interest of the VEGF gene was amplified using appropriate DNA primers by PCR (see above), and the presence of the polymorphisms was identified using restriction enzyme digestion. The following polymorphisms were identified: A-141C; C-460T; C+405G (measured from transcription start).

The results of analysis of genotype and allelic frequency for C-460T are represented below ((%)n):

Table 3: Correlation between polymorphism –460 and Ischaemic Heart Disease

		Normal	Ischaemic
Allele	C	61 (57%)	59 (38%)
	T	45 (43%)	95 (62%)
Genotype	CC	17 (32%)	8 (10%)
	CT	27 (51%)	43 (56%)
	TT	9 (17%)	26 (34%)

The C-460T genotype differed significantly between normal and ischaemic subjects ($\chi^2 = 11.1$, $p = 0.004$). The T allele was significantly over-represented in the ischaemic group ($p = 0.002$), and was associated with an odds ratio of 2.2 (95%CI 1.3-

3.6) for the presence of atheromatous coronary artery disease. A logistic regression model confirmed that the T allele at –460 remained a significant independent predictor of ischaemic heart disease (p = 0.001, R = 0.22, OR = 3.1 (95%CI 1.5 – 6.2) after controlling for possible confounding variables (age, sex, a history of hypertension, prior MI, smoking status and a family history of ischaemic heart disease). These data illustrate that subjects with a –460T genotype are more susceptible to ischaemic heart disease.

Therefore diagnosis, or prediction of a predisposition to, heart disease may be aided by identifying this polymorphism in DNA samples according to the method of the first aspect of the invention.

There are growing numbers of patients with ischaemic heart disease who have intractable symptoms of angina pectoris and who are not suitable for angioplasty or surgical revascularisation. Screening for these polymorphisms will identify individuals at risk before their symptoms become so severe.

2.2.2 STEROID SENSITIVE NEPHROTIC SYNDROME IN CHILDREN

Correlation between + 405 and – 460 polymorphisms was also established with the incidence of steroid sensitive nephrotic syndrome in children.

By way of example, statistical analysis was performed to evaluate the differences in incidence of the - 460 polymorphism in a first population of normal healthy controls and a second population with Nephrotic Syndrome. The data in Table 3 illustrate that the TT genotype was more prevalent in Nephrotic Syndrome. This information may be used by a clinician in the diagnosis of the syndrome, predicting a risk of developing the syndrome and also in making a prognosis.

Table 4: Correlation between polymorphism –460 and Nephrotic Syndrome

		Normal Healthy Controls (N = 115)	Nephrotic Syndrome (N = 59)	
Allele	C	53% (121)	49% (58)	
	T	47% (109)	51% (60)	p = 0.5721
Genotype	CC	24% (28)	32% (19)	p = 0.2838
	CT	57% (65)	34% (20)	p = 0.0063
	TT	19% (22)	34% (20)	p = 0.0396

2.2.3. RHEUMATOID ARTHRITIS (RA)

Significant differences were identified in the prevalence of the – 460 and + 405 polymorphisms in RA and non-RA populations.

Blood was drawn from subjects and the area of interest of the VEGF gene was amplified using appropriate DNA primers by PCR (see above) and the presence of the polymorphisms was identified using restriction enzyme digestion.

(i) The -460 polymorphism

The results of analysis of genotype and allelic frequency for C-460T are represented below ((%)n):

Table 5: Correlation between polymorphism –460 and RA

		Non-RA (N = 45)	RA (N = 43)		
Allele	C	46% (41)	59% (50)		P = 0.0949
	T	54% (49)	42% (36)		
Genotype	CC	18% (8)	37% (16)	**P = 0.0408**	
	CT	56% (25)	42% (18)	P = 0.1989	P = 0.1230
	TT	27% (12)	21% (9)	P = 0.5280	

(ii) The +405 polymorphism

The results of analysis of genotype and allelic frequency for G+405C are represented below ((%)n):

Table 6: Correlation between polymorphism + 405 and RA

		Non-RA Controls (N = 42)	RA (N = 33)		
Allele	G	65.5% (55)	82% (54)		P = 0.0885
	C	34.5% (29)	18% (12)		
Genotype	GG	43% (8)	67% (22)	**P = 0.0402**	
	GC	45% (25)	30% (10)	P = 0.1874	**P = 0.0258**
	CC	12% (12)	3% (1)	P = 0.1597	

2.2.4. OVARIAN CARCINOMA

Significant differences were identified in the prevalence of the – 460 polymorphism in patients with ovarian carcinoma and normal populations.

Blood was drawn from subjects and the area of interest of the VEGF gene was amplified using appropriate DNA primers by PCR (see above) and the presence of the polymorphisms was identified using restriction enzyme digestion.

The -460 polymorphism

The results of analysis of genotype and allelic frequency for C-460T are represented below ((%)n):

Table 7: Correlation between polymorphism –460 and Ovarian Cancer

		Normal Healthy Controls (N = 115)	Ovarian Cancer (N = 29)		
Allele	C	53% (121)	62% (36)		P = 0.2381
	T	47% (109)	38% (22)		
Genotype	CC	24% (28)	49% (13)	**P = 0.0383**	
	CT	57% (65)	35% (10)	**P = 0.0390**	P = 0.0608
	TT	19% (22)	21% (6)	P = 0.7986	

EXAMPLE 3

Further detailed experiments were performed to correlate specific polymorphisms with ischaemic heart disease. Example 2 had established a linkage with the -460 polymorphism tests were also performed with the +405 and -141 polymorphisms as comparative examples.

3.1 METHODS

3.1.1 Patient population

The patient population was enrolled from a tertiary referral cardiology unit in Sheffield, in the North of England. Patients were exclusively Caucasian, and were enrolled from patients attending for diagnostic cardiac catheterisation, either for suspected ischaemic heart disease, or for evaluation of valvular heart disease, congenital heart disease or primary myocardial disease.

Coronary angiograms were categorised as abnormal if ≥ 1 epicardial coronary artery contained a stenosis of ≥ 30% luminal area of the artery. Angiograms were examined visually by two independent cardiologists. Normal coronary arteries were defined as either smooth-walled vessels or vessels containing mural irregularities which caused < 30% reduction of lumen diameter.

Clinical characteristics of the patients and angiographically normal controls were collected at the time of enrolment; specifically, a personal history of hypertension, diabetes mellitus, cigarette smoking and a family history of ischaemic heart disease, in addition to age and gender.

3.1.2 Genotyping

Genomic DNA was extracted from anticoagulated blood using standard methods. Sample DNA was typed for the A-141C, G+405C and C-460T polymorphisms using PCR amplification and restriction enzyme digestion of the PCR products as previously described (see Example 2). The polymorphisms were read from the gel images by an observer blinded to the disease status of the patients.

3.1.3 Statistical analysis

The association between the presence or absence of ischaemic heart disease with each genotype and the presence of individual alleles was examined using

Fisher's Exact Test or calculation of the χ^2 value. Independence of associations detected from possible clinical confounders was determined by forward stepwise logistic regression modelling.

3.2 RESULTS

A total of 384 subjects were analysed; 268 (69.8%) had ischaemic heart disease, and 116 (30.2%) had normal coronary arteries on angiography. The clinical characteristics of the study population are shown in Table 8. There was an excess of males in the disease group, as would be expected.

Table 8: Clinical characteristics of the study population.

	Normal subjects	IHD	p
N	116	268	
Age (SD)	56.7 (12.2)	57.8 (14.6)	ns
Sex (m:f)	49:67	214:54	0.0001
Diabetes, n(%)	6 (5)	17 (6)	ns
Current or ex-smokers, n(%)	62 (53)	156 (58)	ns
Family history of IHD, n(%)	45 (39)	82 (31)	ns
Hypertension, n(%)	34 (29)	73 (27)	ns

The allelic frequencies for the three polymorphisms are shown in Table 9. The C-460T polymorphism showed a trend towards a higher frequency of the C allele in angiographically normal patients ($p = 0.08$). This gave an odds ratio of 0.77 (95% CI 0.56 – 1.04), suggesting a reduced risk of 0.75 for ischaemic heart disease in subjects with the C allele at –460. The prevalence of the three promoter polymorphism genotypes is shown in Table 3. Homozygosity for the C allele at –460 was significantly associated with the non-ischaemic phenotype (frequency of 0.28 in normals vs. 0.17 in subjects with ischaemic heart disease, $p = 0.013$). The odds ratio for ischaemic heart disease for individuals with the CC genotype was 0.51 (95% CI

0.3 – 0.85). No association was found for the presence of ischaemic heart disease and the A-141C and the G+405C polymorphisms.

Table 9: Allelic frequency of the three polymorphisms in normal and ischaemic populations. IHD – ischaemic heart disease

	A-141C		G+405C		C-460T	
	A	C	G	C	C	T
IHD	0.91	0.09	0.66	0.34	0.43	0.57
Normal	0.88	0.12	0.64	0.36	0.50	0.50
P	ns		ns		0.08	

Table 10: Prevalence of the three polymorphisms in normal and ischaemic populations. IHD – ischaemic heart disease.

Polymorphism	Genotype	IHD	Normal	p
A-141C	AA	87% (232)	80% (93)	0.20
	AC	9% (25)	15% (18)	
	CC	4% (11)	4% (5)	
G+405C	GG	40% (107)	40% (47)	0.25
	GC	52% (140)	46% (54)	
	CC	8% (21)	13% (15)	
C-460T	CC	17% (45)	28% (33)	0.03
	CT	53% (143)	43% (50)	
	TT	30% (80)	28% (33)	

The independence of the association found for C-460T and coronary artery disease from likely confounding variables was investigated by construction of a logistic regression model, where the presence or absence of ischaemic heart disease was entered as the dependent variable, and a history of hypertension, diabetes mellitus, a family history of ischaemic heart disease, sex and smoking (never vs. current | ever), were entered as explanatory covariates. Age was also entered as an independent variable. The presence of the CC genotype at –460 remained a significant independent negative predictor of the ischaemic phenotype (β = -0.51, R = -0.13, p = 0.003, OR = 0.60, 95% CI 0.43 – 0.83). Male sex was a strong positive predictor of the ischaemic phenotype (β = 0.92, R = 0.31, $p < 0.0001$, OR = 2.5, 95% CI 1.9 – 3.3). A prior history of myocardial infarction was also an independent predictor (β = 0.91, R = 0.25, $p < 0.0001$, OR = 2.5, 95% CI 1.8 – 3.5).

These data show that there is a significant association between the CC genotype of the C-460T polymorphism and the absence of obstructive coronary disease (p = 0.013). Furthermore, this association remains after controlling for other possible confounding risk factors, with homozygosity of the C allele at C-460T having an odds ratio of 0.6 (95% CI 0.43 – 0.83) for the presence of ischaemic heart disease. This suggests that subjects with the CC genotype are over a third less likely to show demonstrable coronary arterial disease than those with the CT or TT genotype. This finding has important implications for the aetiology of ischaemic heart disease.

EXAMPLE 4

Examples 1 identified polymorphisms whereas Examples 2 and 3 established correlations between particular polymorphisms and specific diseases or disorders. In the light of the inventors experimental findings.

A test according to the method of the first aspect of the invention was designed for use in a clinical or testing laboratory. The inventors have found that the method of the invention may preferrably be carried out using the following protocols:

4.1 PROTOCOLS

4.1.1 DNA Extraction from Whole Blood or Granulocytes.

1. Collect 2 - 4ml of blood in EDTA tubes (preferably sodium salt). Samples may be stored at -20°C before processing. However once thawed samples should be processed without delay. Larger volumes of blood can be processed but volumes of all solutions must be scaled up appropriately.
2. For whole blood and granulocyte fractions add 9ml of cold lysis buffer 1 (see A below). Mix / rotate for 15 min. For cultured cell pellets start at step 6.
3. Collect the nuclei by centrifugation at 3,000rpm for 10 min. at room temperature (brake off).
4. Pour off the supernatant then wash the pellet by addition of 9ml of lysis buffer 1 and centrifuge at 3,000rpm for 10 min. at room temp. Discard the supernatant and then vortex the pellet to disrupt it.
5. Resuspend the pellet in 3ml of lysis buffer 2 (see B below) and lyse the pellet completely by pipetting.
6. Add 1ml of 10M ammonium acetate, vortex the sample vigorously then centrifuge at 3,000rpm for 20 min. at room temp (brake off).
7. Remove the supernatant to a clean tube containing 3ml of propan-2-ol (iso-propanol) and mix gently by inversion.
8. Centrifuge at 3,000rpm for 10 min. then discard the supernatant.
9. Wash the resulting DNA pellet by resuspension in 3ml of 70% ethanol. Either hook out the DNA into a microfuge tube or centrifuge again at 3,000rpm for 10 min. and discard the supernatant. Allow the pellet to air dry.
10. Redissolve the DNA in up to 500µl dH_2O and measure the concentration.

A :- Lysis Buffer 1

	final concentration	for 1 litre
Ammonium Chloride	155mM	8.3g
Potassium Hydrogen carbonate	10mM	1g
0.5M EDTA	1mM	2ml

B :- Lysis Buffer 2

	final concentration	for 100ml
0.5M EDTA	25mM	5ml
10% SDS	2%	20ml

4.1.2 PCR Amplification

1. Amplifications were carried out in a 20µl reaction containing 100ng of genomic DNA;

The reaction vessels contained:

10pmoles of primers [5' ATTTATTTTTGCTTGCCATT 3' (SEQ ID No. 1) and 5' GTCTGTCTGTCTGTCCGTCA 3' (SEQ ID No. 2) for the +405 polymorphism and primers 5' TGTGCGTGTGGGGTTGAGCG 3' (SEQ ID No. 5); and 5' TACGTGCGGACAGGGCCTGA 3' (SEQ ID No. 4) for the –460 polymorphism]; 0.75mM of each dNTP; 10% DMSO; 4mM and 5mM $MgCl_2$ (for +405 and –460 respectively); 16.6mM $(NH_4)_2SO_4$; 67mM Tris.HCl pH 8.0; 85mg/ml BSA and 0.5U of *Taq* DNA polymerase.

2. Samples were denatured at 95°C for 3 min. followed by 32 cycles at 95°C for 1 min., 55 or 60°C* for 1 min. (* for +405 and –460 respectively), 72°C for 1min., with a final extension step of 72°C for 5 min.

4.1.3 Restriction Enzyme Digestion of PCR Products and Agarose Gel Electrophoresis of DNA Fragments

1. Monitor 5µl of each PCR product on an agarose gel. If amplification is satisfactory proceed to digestion.

2. To each sample add :-

10 x restriction buffer	2 µl
BSA (U/ µl)	0.2 µl
restriction enzyme*	2 - 5 units (i.e. 0.2 - 0.5µl at 10U/µl)
dH_2O	to 5µl

(* BsmFI for +405 and BstUI for –460)

this can be prepared as a master mix mix each sample and centrifuge briefly to collect.

3. Incubate the samples at the prescribed temperature for the particular restriction enzyme for 2-3 hours.
4. To determine the restriction digest pattern for each sample add 4µl of 5 x TBE loading dye (see C below) to each sample. Then electrophorese the samples on a 3% 1 x TBE agarose gel containing ethidium bromide at 0.5µg/ml in 1 x TBE buffer (see D below) and include a marker such as 100bp ladder (GIBCO-BRL).
5. Visualise the gel on a UV transilluminator and then photograph the gel.

C :- 5 X TBE loading dye

	final concentration	for 20ml
10 X TBE	5 X	10ml
glycerol	30%	6ml
bromophenol blue	0.25%	50mg
xylene cyanol	0.25%	50mg
dH_2O		4ml

D:- 10 x TBE

	final concentration	for 2 litres
Tris	0.89M	216g
Orthoboric acid	0.89M	110g
0.5M EDTA	20mM	80ml
dH_2O		up to 2 litres

4.1.4 Genotyping of Test Samples

(i) For the +405 Polymorphism

A DNA fragment of 304 bp in size corresponds to a C allele

DNA fragments of 193bp and 111bp in size correspond to a G allele

Test samples having only a 304bp fragment possess a CC homozygous genotype

Test samples having only 193bp and 111bp fragments possess a GG homozygous genotype

Test samples having 304bp, 193bp and 111bp fragments possess a CG heterozygous genotype

(ii) For the -460 Polymorphism

A DNA fragment of 175bp in size corresponds to a T allele

DNA fragments of 155bp and 20bp in size correspond to a C allele

Test samples having only a 175bp fragment possess a TT homozygous genotype

Test samples having only 155bp and 20bp fragments possess a CC homozygous genotype

Test samples having 175bp, 155bp and 20bp fragments possess a TC heterozygous genotype

4.2 RESULTS

(i) For the +405 Polymorphism

A GG homozygous genotype indicates that an individual may be predisposed to development of rheumatoid arthritis.

(ii) For the -460 Polymorphism

A CC homozygous genotype indicates that an individual may be predisposed to development of rheumatoid arthritis.

A CC homozygous genotype indicates that an individual may be predisposed to development of ovarian cancer.

A CC homozygous genotype indicates that an individual may be protected from the development of ischaemic heart disease.

A TT homozygous genotype indicates that an individual may be predisposed to development of nephrotic syndrome.

EXAMPLE 5

According to a preferred embodiment of the invention a kit is formed to be used in clinical or testing laboratories.

A kit containing the following components was formed for use according to the invention:

A) **PCR primers -**

(i) For +405 polymorphism genotyping

5' ATTTATTTTTGCTTGCCATT 3' (SEQ ID No. 1)

5' GTCTGTCTGTCTGTCCGTCA 3' (SEQ ID No. 2)

or

(ii) For –460 polymorphism genotyping

5' TGTGCGTGTGGGGTTGAGCG 3' (SEQ ID No. 5)

5' TACGTGCGGACAGGGCCTGA 3' (SEQ ID No. 4);

B) Protocols for PCR amplification, restriction enzyme digestion of PCR products and agarose gel electrophoresis of DNA fragments (e.g. See Example 4).

C) Control DNA samples of known genotype for each polymorphism.

D) Information on genotyping and the significance of the results (see Example 4).

The Kit may also contain relevant buffers (e.g. A- D from Example 4). These may be in liquid form and preferably provided as pre-measured aliquots. Alternatively the buffers may be in concentrated (or even powder form) for diluting.

The Kit may further comprise suitable reaction vessels, centrifuge tubes etc.